

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
1 May 2003 (01.05.2003)

PCT

(10) International Publication Number
WO 03/035208 A1

- (51) International Patent Classification⁷: **B01D 9/00**, G01N 1/00, 33/00, 21/76
- (21) International Application Number: PCT/US02/33890
- (22) International Filing Date: 23 October 2002 (23.10.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/344,581 23 October 2001 (23.10.2001) US
- (71) Applicant: **UAB RESEARCH FOUNDATION** [US/US]; 701 South 20th Street, Suite 1120-G, Birmingham, AL 35294 (US).
- (72) Inventors: **CHAIT, Arnon**; 408 Glen Park Drive, Bay Village, OH 44140 (US). **DeLUCAS, Lawrence**; 2739 Altadena Road, Birmingham, AL 35294 (US). **STOOPS, Brad**; 28041 Lincoln Road, Bay Village, OH 44140 (US). **BELGOVSKIY, Alexander**; 26101 Miles Road, Cleveland, OH 44128 (US).
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.
- (74) Agents: **JOHNSON, William, R.** et al.; Needle & Rosenberg, P.C., The Candler Building, 127 Peachtree Street N.E., Atlanta, GA 30303-1811 (US).
- Published:**
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/035208 A1

(54) Title: METHOD FOR PREPARATION OF MICROARRAYS FOR SCREENING OF CRYSTAL GROWTH CONDITIONS

(57) Abstract: A method of screening solution conditions suitable for the crystallization of macromolecules with a picogram to a microgram of protein using picoliter or nanoliter volumes is provided. A preferred method comprises preparing a plurality of recipe solutions in milliliter volumes, aspirating protein and recipe solutions, respectively, from a plurality of wells, and dispensing the protein and recipe solutions into a plurality of wells, covering the combined protein and recipe solutions with oil, and maintaining the solution wells until crystallization or precipitation occur therein.

ATTORNEY DOCKET NO.21085.0035P1**METHOD FOR PREPARATION OF MICROARRAYS
FOR SCREENING OF CRYSTAL GROWTH CONDITIONS****CROSS REFERENCE TO RELATED APPLICATIONS**

5

This application is related to, and claims the benefit of priority of, Application Serial Number 60/308,698, filed July 30, 2001, pending, Application Serial No. 09/947,665, filed September 6, 2001, pending, Application Serial No. 10/208,576, filed July 30, 2001, pending, which claimed priority from Provisional Application Serial No. 60/308,698, filed July 30, 2001, and Provisional Application Serial No. 60/328,958, filed October 12, 2001, and Provisional Application Serial No. 60/344581, filed October 23, 2001, pending, which applications are hereby incorporated by this reference in their entireties.

15

BACKGROUND**FIELD OF THE INVENTION**

The present invention generally relates to the crystallization of macromolecules such as proteins from solutions. The present invention is particularly related to the preparation of arrays of solutions useful for screening crystallization conditions to determine which conditions are optimal for the crystallization of macromolecules. Even more particularly, the present invention is related to preferred techniques of preparing solutions suitable for screening, and for the screening conditions themselves, where the total macromolecule solution volume is very small, typically in nanoliter quantities or below.

BACKGROUND OF THE INVENTION

The crystallization of macromolecules, especially of biological macromolecules, is an important activity in many fields. Obtaining high quality crystals of any given macromolecule typically enables subsequent solution of the

ATTORNEY DOCKET NO.21085.0035P1

macromolecule's three dimensional structure (atomic configuration) using diffraction techniques. Of particular interest, the three dimensional structures so obtained can be of paramount importance in the rational design of drugs or other therapeutics. Additionally, it is commonly accepted that one of the primary benefits

5 that will flow from elucidation of the genome will be an improved understanding gained of the proteome, the entire set of expressed proteins in a particular biological organism. However, the full advantage that can be gained from that improved understanding of the proteome can only be realized with the knowledge of the three dimensional atomic configuration of each substituent protein. For example, a

10 knowledge of the three dimensional atomic configuration of a given protein, referred to herein as its active conformation, will provide opportunities to use computerized methods and techniques, among others, to design drug molecules that will effectively and/or efficiently interact with the target protein.

The orderly crystallization of macromolecules from a solution containing the

15 macromolecules results from complex interplay between many variables, including process kinetics (the rate at which the solution approaches supersaturation conditions), pH, ionic composition of buffer components in the solution, the type and concentration of crystallization agents, temperature, etc. As a result, the process of determining what conditions are suitable for crystallizing a given macromolecule

20 is formidable. Typically, hundreds or thousands of experiments are conducted in which one or more of these variables is different from those of one or more of the others. This process, often referred to as screening, is useful to identify the best conditions to use in order to obtain high quality crystals. Usually, once screening has identified suitable conditions, further experiments are performed with those

25 conditions so identified to obtain large crystals for subsequent diffraction studies.

The advent of the genomics and proteomics age has resulted in an unprecedented increase in the number of biological macromolecules available for crystallization. However, due to technical difficulties in expressing and purifying many proteins, the amount of protein available for screening crystallization

30 conditions is often not adequate to provide for screening a suitable number of

ATTORNEY DOCKET NO.21085.0035P1

crystallization conditions if conventional methods of screening are used. This can be illustrated by typical circumstances encountered where only sub-milligram amounts of proteins are available, but it is desirable to screen hundreds or thousands of different conditions. For screening such a large number of conditions with such a small amount of a protein, each screening experiment must use no more than a nanogram of protein. Ideally, each screening experiment would use even less protein. As typical supersaturation levels of proteins in solution are much higher than those corresponding to small molecular weight compounds, the volume of the solution in which the experiment must be carried out, in order to achieve supersaturating concentrations, must be very small. Examples of the small volumes required are typically less than or equal to a few hundred, a hundred, fifty, twenty-five, ten, five or one nanoliter(s).

Conducting screening experiments of macromolecules, in particular of those biological macromolecules, are further constrained by factors in addition to the quantity of protein required. These further constraints include those related to the types of solutions used, the accuracy requirements, the means necessary to dispense small volumes of various components, and limitations on the techniques that can be used for practical manipulation of small volumes. Each of these additional constraints must be overcome in order to allow the screening in low volumes to lessen the quantity of protein required. It would be further desirable to provide such methods that address each of these particular constraints in a manner that allows high levels of flexibility, accuracy, precision and reproducibility. Furthermore, it would be an advantage if the methods so provided were simple to use.

25

SUMMARY OF THE INVENTION

In accordance with the purpose(s) of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to methods for the screening of crystallization conditions in small solution volumes. In particular, the method

ATTORNEY DOCKET NO.21085.0035P1

provides improvements that resolve certain technical aspects that impede the use of arrays of experimental conditions.

In one aspect of the present invention, solutions used in the crystallization screens, such as protein, buffer or precipitant solutions, can be prepared in larger
5 volumes than are used in each individual crystallization screening reaction. The preparation and mixing of the solutions using water, buffers, and various crystallization agents can be performed using automated robotic dispensing systems. The volume of the solutions prepared can be greater than 10, 50, 100, 200, 300, 500, 750 or 1000 microliters. The volume of the solutions prepared can also be less
10 than 50, 100, 200, 300, 500, 750, 1000 or 2000 microliters. The solutions, once prepared, can be stored for a period of time prior to their use in the crystallization condition screening experiments. Alternatively, the solutions can be prepared as needed prior to their use in the crystallization screening experiments.

The solutions can be prepared, and/or stored in multi-well plates as are
15 known to those of skill in the art. Examples of these multi-well plates include, but are not limited to, 96 well plates and 384 well plates. The sets of solutions prepared are referred to herein as recipe libraries.

The individual solutions themselves, also referred to herein as recipes or recipe solutions, can be more dilute than is typical in crystallization conditions used
20 in normal, larger volume formats. For example, it is typical in the art to provide solutions that contain a greater percentage of volatile solvent than is required and/or which allows precipitation and/or crystallization. As is understood by those of skill in the art, for precipitation and/or crystallization to occur, evaporation of some volume of the solvent is required. Alternatively, the removal of solvent by some
25 other method (e.g., absorption, dialysis, or other methods as are known to those of skill in the art) with the consequent concentration of protein and or precipitant agent can substitute for loss of solvent by evaporation.

The present invention includes the use of protein solutions, precipitant solutions, and recipe solutions containing greater percentages of volatile solvent
30 and/or lower concentrations of constituents required for precipitation and/or

ATTORNEY DOCKET NO.21085.0035P1

crystallization than is used by those processes that have been used. If the volume of solvent at the point at which crystallization occurs is 100%, the volume of solvent present in the recipes is greater than 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 220, 250, 300, 350, 400, 450 or 500%. The volatile solvent can be water or
5 other solvents as are known to those of skill in the art. Other solvents can be mixtures of more than one solvent, composition or compound.

In another aspect, the system provided by the present invention includes the use of an automated fluid manipulation system. The automated fluid manipulation system can take recipe solutions from a recipe library and transfer these to other
10 formats. This reformatting can be done to create different recipe libraries or can be used to replicate large numbers of low-volume libraries in multiwell plates. It is contemplated that these multiwell plates can be adapted for use by automated and/or high-throughput machinery other than that machinery described herein. The automated fluid manipulation system of the present invention can include multiple
15 dispensing tips arranged on a single dispense head or can include single or multiple dispensing tips arranged on more than one dispense head. For example, the system can include 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 32, 40, 48, 56, 64, 72, 80, 88 or 96 dispensing tips. The tips can be arranged on one, two, three, four, five, six or eight dispensing heads.

20 In yet another aspect, the invention provides an automated crystallization system. The provided system can take up a fluid component from a selected volume, and can also dispense a fluid component to a selected volume. In particular aspects, the machinery of the invention can aspirate and dispense protein solution droplets and/or recipe solution droplets into at least 10, 20, 50, 100, 200, 400, 600 or
25 1000 wells in a multi-well plate. The volume of the protein and/or recipe solutions that can be dispensed can be less than 1, 2, 5, 10, 20, 30, 50, 75, 100, 150, 300, 500 or 1000 nanoliters. The volume of the protein and/or recipe solutions that can be dispensed can be greater than 10, 20, 30, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 picoliters, and greater than 2, 3, 5, 10, 20, 30, 50, 75, 100, 150,
30 300, 500, 700 or 900 nanoliters. Once volumes of proteins and/or recipe solutions

ATTORNEY DOCKET NO.21085.0035P1

are dispensed into a plurality of wells in a multi-well plate, the respective solutions in the wells can be quickly covered with an oil mixture. Optionally, the entire well plate, or portions of the well plate can be covered with a covering. Examples of a covering include, but are not limited to, those made from plastic compositions such as tape. If tape is used, the tape can be a polymer such as, but not limited to, Teflon. Optionally, the plate can be cooled below room temperature to minimize or control evaporation. Optionally, the relative humidity of the environment within which the plate is contained can be raised to minimize or control evaporation.

In a particular embodiment of the invention, the crystallization system includes a first recipe solution preparation and storage sub-system used to prepare a plurality of recipe solutions for storage until the recipe solutions are required for use within the crystallization system, and a second aspiration and dispensing sub-system used to aspirate and dispense a plurality of protein solutions and of the recipe solutions, respectively, into a plurality of wells.

In another embodiment, a crystallization experiment preparation system can include: a dispense head provided with plurality of fluid dispenser tips; a video camera and a long range microscope each carried on said dispense head; a stage constructed and arranged to move the dispense head, the video camera, and the long range microscope together in the X, the Y, and the Z directions, respectively; a deck area positioned with respect to the stage, said deck area having at least one fixture for receiving at least one recipe plate, at least one protein plate, at least one crystallization plate, a quality control area, and a wash system, respectively, thereon.

In another embodiment, a crystallization experiment preparation system can include: a dispense head provided with plurality of fluid dispenser tips; a video camera and a long range microscope each carried on said dispense head; a deck area positioned with respect to the dispense head, said deck area having at least one fixture for receiving at least one recipe plate, at least one protein plate, at least one crystallization plate, a quality control area, and a wash system, respectively, thereon, wherein the dispense head, the video camera, and the long range microscope are held together in a stationary position with respect to a deck area; and wherein the deck

ATTORNEY DOCKET NO.21085.0035P1

area is constructed and arranged to be moved with respect to the dispense head, the video camera, and the long range microscope by a stage movable in the X, the Y, and the Z, directions, respectively.

In another embodiment, a crystallization experiment preparation system can include: a dispense head provided with plurality of fluid dispenser tips; a video camera and a long range microscope each carried on said dispense head; a first stage on which the dispense head is positioned, said first stage being constructed and arranged to move the dispense head in the Z direction; a deck area positioned with respect to the dispense head, said deck area having at least one fixture for receiving at least one recipe plate, at least one protein plate, at least one crystallization plate, a quality control area, and a wash system, respectively, thereon; and a second stage adapted to carry said deck area in the X and the Y directions, respectively.

The protein and/or recipe solutions provided can be used for screening crystallization conditions using a number of different methods of crystallizing proteins from solutions. These methods include, but are not limited to, batch crystallization wherein the protein and precipitant-containing recipe solutions are mixed together, and those methods wherein the protein solution and precipitant-containing solution, recipe solutions, are kept physically separate but in fluid communication with one another. Methods wherein the fluid communication is liquid-liquid, liquid-vapor or vapor-vapor are therefore contemplated.

In a particular embodiment, the method for screening protein crystal growth conditions can include: providing a microarray with a plurality of wells in the microarray; dispensing a volume of protein solution containing a protein into at least one of the wells; dispensing a recipe solution into at least one of the wells, wherein the recipe solution was prepared from bulk ingredients prior to being dispensed into the wells and was stored in a plurality of containers or in a second multiwell plate; subjecting the protein solution, or a combination solution formed by the combination of the protein solution with the recipe solution, to environmental conditions effective to form protein crystals; and observing the growth of protein crystals or precipitation of protein.

ATTORNEY DOCKET NO.21085.0035P1

In another particular embodiment, the method for screening protein crystal growth conditions can include: providing an array of at least two wells; dispensing a volume of protein solution containing a protein into at least one of the wells; dispensing a recipe solution into at least one of the wells, wherein the recipe solution
5 was prepared from bulk ingredients prior to being dispensed into the wells and was stored in at least one of a plurality of containers or in a second multiwell plate; subjecting the protein solution, or a combination solution formed by the combination of the protein solution with the recipe solution, to environmental conditions effective to form protein crystals; and observing the growth of protein crystals or precipitation
10 of protein.

In one preferred method, referred to as the modified vapor diffusion process, the protein solution is placed in a second well near a first well. The first well is filled with a recipe solution and the second well is filled with a mixture of the protein solution and a smaller amount of the recipe solution than is present in the
15 first well. A mixture of oils can be used to cover the first and/or second wells. The plate in which the first and second wells, respectively, or any number of first and second well pairs, are formed can be sealed with a clear cover or tape. Each well pair can be interconnected with a channel providing for vapor contact between each pair of first and second wells.

20 In another preferred method, an under-oil process, solutions in wells are overlaid with an oil, mixture of oils, or composition containing oil. Solutions so overlaid, or wells containing such solutions, or plates containing wells can be further sealed with a cover. The cover used can be a clear cover. The cover used can be tape.

25 In another aspect, the invention provides for the proper sequence with which the recipe and protein solutions are dispensed. In a preferred method, the protein solution is dispensed prior to, simultaneously with, or after the recipe solution is dispensed. If the dispensing of the protein and recipe solutions is not substantially simultaneous, i.e., separated by less than 1, 2, 3 or 5 minutes, the dispensing can be
30 separated by greater than 1, 2, 3, 5, 10, 15, 20, 30, 60, 90, 240, 360, 480, 720, or

ATTORNEY DOCKET NO.21085.0035P1

1200 minutes, or may be separated by less than 2400, 1200, 720, 480, 360, 240, 90, 60, 30, 20, 15, 10, 5, 3 or 2 minutes.

In still another aspect, the invention provides a method by which accurate dispensing and formulation of solutions is achieved. In a specific embodiment, accurate dispensing of volumes is achieved by use of larger dispensed volumes.

In another aspect, the invention provides a method used to control the initial rapid evaporation rate from the droplets.

In another aspect, the invention provides for the slow evaporation rate that is required for the orderly growth of crystals.

One particularly important aspect of the present invention is the use of dilute solutions which require significant loss of solvent prior to crystallization occurring.

Additional advantages of the invention will be set forth in part in the description which follows or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only, and are not restrictive of the invention, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate (one) several embodiment(s) of the invention and together with the description, serve to explain the principles of the invention.

Figure 1 is a depiction of the recipe solutions preparation system, the RecipeMaker device. The RecipeMaker in this figure is comprised of a robotic liquid handler with 8 probes, 8 computer-controlled syringes, and 8 computer-controlled distribution valves. Computer control of the devices is achieved by use of

ATTORNEY DOCKET NO.21085.0035P1

5 computer with custom control software directing the actions to be taken by the devices of the invention.

Figure 2 is a depiction of the crystallization experiment preparation system, the NanoScreen device. The NanoScreen device is comprised of a computer controlled XYZ stage, a custom fast solenoid valve dispensing system and computer control software and computer control devices that direct action of the dispensing system.

Figure 3 is a depiction of the NanoScreen device showing the deck area. This close-up depiction of the deck area shows an exemplary arrangement of various plates, wash stations, and a quality control area of the device of the invention.

15 Figure 4 is a depiction of the NanoScreen device showing the deck and dispense head. The close-up depiction clearly shows the dispensing tips array above the plates used in the practice of the invention.

Figure 5 is a depiction of the NanoScreen device showing the dispense head as mounted on the computer-controlled Z-axis stage.

20 Figure 6 is a depiction of certain aspects of the NanoScreen device adapted for cleaning the protein and recipe solution tips. This depiction illustrates features of the protein and recipe solution tip wash stations.

Figure 7 depicts crystals of concanavalin-A protein formed in a droplet prepared by the RecipeMaker and NanoScreen devices. The droplet contained a total of 45 nanoliters of protein and recipe solution. Crystals appeared two days after preparation of the droplet after incubation at room temperature. Crystals were photographed using video microscopy.

Figure 8 depicts crystals of thaumatin protein formed in a droplet prepared by the RecipeMaker and NanoScreen devices. The droplet contained a total of 30 nanoliters of protein and recipe solution. Crystals appeared after approximately one day after preparation of droplet and incubation at room temperature. Crystals were photographed using video microscopy.

Figure 9 depicts crystals of thaumatin protein formed in a droplet prepared by the RecipeMaker and NanoScreen devices. The droplet contained a total of 45

ATTORNEY DOCKET NO.21085.0035P1

5 nanoliters of protein and recipe solution. The crystals shown formed three days after preparation of the droplet and incubation at room temperature. Crystals were photographed using video microscopy.

Figure 10, parts A-O, depicts crystals of selected proteins formed in droplets prepared by use of the RecipeMaker and NanoScreen devices. Droplets depicted
10 contained a total of 80 nanoliters of protein and recipe solution. The protein solution in each drop varied between 20 nanoliters and 51 nanoliters. The remainder volume necessary to bring the total volume to 80 nanoliters in each case was added solution containing buffer and precipitants.

15 **DETAILED DESCRIPTION**

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein, and to the Figures and their previous and following
20 description.

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that this invention is not limited to specific methods, specific solutions, or to particular devices, as such may, of course, vary. It is also to be understood that the terminology used herein is for the
25 purpose of describing particular embodiments only and is not intended to be limiting.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a precipitant" includes mixtures of a precipitant,
30 reference to "a solution" includes combination of and/or mixtures of two or more such solutions, and the like.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular

ATTORNEY DOCKET NO.21085.0035P1

5 value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

10 In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

15 One method provided by the present invention is an improvement over previous methods for screening of plurality of solution conditions in low volumes to determine those conditions that lead to crystallization of macromolecules, and in particular, of biological macromolecules. Preferably, the quantity of the biological macromolecule that is used for each experiment is in the microgram, nanogram, or
20 picogram range, and the preferred volume of the solution is in the picoliter or nanoliter range. It will be appreciated by those skilled in the art that the particular volumes and amounts of protein or solution ingredients employed will vary without limitation according to the protein, its concentration, and the desired experimental protocol. As used herein, the term "macromolecule" is meant to include all naturally
25 occurring and synthetic peptides, polypeptides, proteins, protein complexes, and polymers.

Crystallization of macromolecules from solutions occurs when the concentration of the macromolecule exceeds the maximum concentration allowable in solution that is dictated by the thermodynamics of the macromolecule solution.

30 While phase transitions from a solution to a solid state always occur with increasing concentration, orderly growth of the solid in a highly order crystalline form does not. Crystal growth of macromolecules is a special form of phase transition that is very difficult to achieve in practice. Consequently, those of skill in the art seeking to promote crystal growth generally conduct multiple experiments with a plurality of

ATTORNEY DOCKET NO.21085.0035P1

5 solution conditions that include variations in the type, strength, and quantity of buffers, the pH, temperature, and the type and quantity of different additives collectively referred to as crystallization agents. The concentrations of these components are often quite low, sometimes less than one percent of the total volume of the solution. For example, surfactants typically used for crystallization of
10 membrane-bound proteins are generally present at very low concentrations. In addition, the concentrations of these components are often quite critical in the success or failure of crystal growth. Normally, when crystallization screening is conducted according to general practices, screening condition trials are prepared by dispensing the various ingredients directly from their bulk solutions forming droplets
15 which are typically of several microliters in volume.

However, when the volume of each trial condition tested is too low, the precision and accuracy required of the dispensing devices used to deliver volumes of each component is greater than that available with current technology. This is particularly true when experiments are conducted in nanoliter range, or lower,
20 volumes and for those experiments wherein solution components are required in low concentrations. For example, trials containing one percent surfactant in a fifty nanoliter droplet require the accurate and repeatable delivery of one half of a nanoliter of 100% surfactant. While certain modern dispensing technologies, in particular those employing piezo driven capillaries, are capable of delivering such
25 volumes accurately and with adequate precision, the wide variation in viscosity and surface tension of solution ingredients results in significant difficulties in realizing the theoretically available precision needed for high throughput low volume experiments.

However, as described herein, the present invention obviates many of the
30 difficulties arising from the differences in surface tension viscosity and the like. Specifically, the present method, by combining larger volumes of components to form the recipe solutions, allows accurate dispensing and formulation of solutions which can then be dispensed in smaller volumes to form the crystallization reactions. Because the differences in the properties of the different recipe solutions are of a

ATTORNEY DOCKET NO.21085.0035P1

5 significantly lower magnitude than the differences in the properties of the component solutions normally dispensed to form crystallization reactions, realizing an appropriately high level of accuracy and precision is attainable by formulating solutions using the presently disclosed method.

10 In one aspect of the present invention, the preparation of the plurality of protein and/or recipe solutions that are subsequently used in crystallization condition screening experiments is separate from the step of actually preparing the crystallization condition trials. A large number of individual solutions representing a plurality of ingredients can be prepared by an automated dispensing system. The dispensing system may comprise a commercially available computerized robotic
15 dispenser in which one or more dispensing probes are connected to reservoirs containing the bulk ingredient solutions. Depending on the number of the bulk solutions required, and the number of available dispensing probes, it is possible that there may be more bulk solutions required than there are dispensing probes. When this is the case, computerized distribution valves can be used to switch among the
20 ingredients, thereby allowing for the dispensing of a greater number of different bulk solutions than the number of dispensing probes.

Computer programs or other electronic media can be used to direct the action of the automated dispensing system used to prepare the protein and recipe solutions. The computer programs and/or electronic media can include data indicating which
25 bulk ingredients are required in each solution, and can command the dispensing system to dispense specified volumes from each tip/valve combination such that the total volume of all ingredients in the each well is the same or as otherwise desired. Using this method, in which the total protein/recipe solution volume is preferably in the milliliter range, provides for less stringent operational requirements from the
30 dispenser to achieve the correct proportions of ingredients in the solution. A plurality of the solutions can be prepared in standard multi-well plates, sealed, and shelved until equilibration occurs (typically a day or less). If quicker equilibration of the solutions is desired, the sealed plates, also referred to as bulk recipe libraries, can

ATTORNEY DOCKET NO.21085.0035P1

- 5 be mixed using mechanical means known to those skilled in the art including, but not limited to, vortexing and shaking.

Because the automated dispensing system of the present invention that is used to prepare the protein and recipe solutions does so in larger, i.e., preferably greater than nanoliter or more preferably greater than microliter volumes, many
10 dispensing technologies can be used effectively with the required level of accuracy and precision. The dispensing technologies, well known to those of skill in the art, include, but are not limited to, positive displacement pumps, aerosol systems, devices using ink-jet technologies, and piezo-electrically actuated dispensing devices. The manufacture and use of such devices are well within the skill and
15 knowledge of those of ordinary skill in the art.

Once prepared by use of the devices and methods of the present invention, the bulk recipe libraries can be further manipulated. Using the same robotic dispenser used to generate the bulk recipe libraries, or a second dispenser if so desired, the bulk recipe libraries can be reformatted into smaller volume multi-well
20 plates. It is preferred that this reformatting be carried-out only after equilibration or mixing of the larger bulk recipe libraries. The smaller volume multi-well plates can be sealed following reformatting. For example, a bulk recipe library, prepared in 2 milliliter 96 deep well plates, can be re-formatted into 80 microliter 384 well plates. One advantage to this procedure is that it allows crystallization experiments to be
25 conducted in volumes significantly smaller than can be prepared, and/or that the dispensing requirements are not as stringent as they would be otherwise. Further, because the bulk recipe library is first re-formatted into a plurality of low volume plates, the exposure of solvents containing volatile ingredients does not inordinately affect the reproducibility between different sample volumes and/or sample plates. In
30 practice, a bulk recipe library is first re-formatted into a plurality of low volumes plates, which are then used as needed and discarded. Devices and methods suitable for reformatting solutions will typically be those that more accurately and precisely dispense small volumes. Examples of such devices and methods, including the factors to be considered in their design and use, are well-known to those of skill in

ATTORNEY DOCKET NO.21085.0035P1

5 the art as is illustrated by commentary entitled, "Liquid Handling Advances Boost
Lab Productivity," published in The Scientist 9[11] on May 29, 1995, which is
incorporated herein by references for its teachings relating to automated fluid
distribution. Other relevant publications and patents relating to aspects of the
technology required for optimization of the present invention include; "Dispensing
10 Systems for Miniaturized Diagnostics" published in IVD Technology in May, 1998;
and "Nanoliter Dispensing published in Drug Discovery & Development in June,
2002; and references cited therein, each of which is incorporated herein by reference
in its entirety for the purpose of describing features of the devices and methods of
the present invention related to accurately and precisely dispensing a plurality of
15 small volumes. Further, automated devices that can be used to practice the present
invention or that can be adapted to practice the present invention can be obtained
from many different manufacturers. Examples of devices that can be used or
adapted for use include, but are not limited to, those available from Hamilton,
Nanoliter, PerkinElmer Life Sciences, Robbins Scientific, Tecan, Cartesian
20 Technologies, Innovadyne, Gilson, and Zymark.

Simultaneously with this procedure of separating the preparation of recipe
solutions and the screening experiments themselves, the advantages of increased
reproducibility and lowered volume requirements result in further advantages, which
include the adaptability of the process to standard robotic dispensers, the
25 minimization of recipe variation due to evaporation of volatile ingredients when re-
formatting is used, and increased flexibility for high throughput operations that are
inherent in the off-line preparation of recipe libraries that may be stored and used as
needed. Examples of related types of devices that utilize the ability to dispense
small volumes to prepare samples are known to those of skill in the art. For
30 examples of these related types of devices, necessary parameters needed for their
effective operation and teachings adequate to allow those of skill in the art to make
and use the devices of the invention, portions of U.S. Patent Nos. 6,296,811;
6,387,273; 6,423,536; and 6,296,673 and all references cited therein relevant to

ATTORNEY DOCKET NO.21085.0035P1

- 5 dispensing devices and automation of dispensing devices, is incorporated herein by reference.

 In the present invention, the crystallization experiments are conducted using an automated robotic dispenser constructed means arranged to quantitatively dispense nanoliter and microliter volume aliquots. A robotic dispenser in
10 accordance with the present invention thus comprises several distinct components. In a preferred embodiment, these components include, but are not limited to, fast solenoid dispensing systems that use a constant back pressure with an array of dispenser tips, switching valve systems to allow for the computerized syringe aspiration of the recipe and protein solutions and the dispensing of the same, an
15 XYZ directional table providing for the exact placement of the dispenser tips, a deck area with designated locations for the protein solution source plate, the recipe library plate, and the experiment plate thereon, as well as, quality control and wash stations for the dispenser tips, machine vision systems comprising a video camera and a long range microscope lens, and computer controller units.

- 20 Dispensing technologies currently available for operations in low solution volumes have distinct limitations for applications that require dispensing arbitrary recipe solutions, each with different properties, as is required for producing solutions in extremely low volumes. Piezo devices, as used herein, are those that utilize the piezoelectric effect to dispense a fluid, namely, they are devices which include a
25 component such that when a voltage is applied across a certain surface of a solid, the solid undergoes a mechanical distortion. For example, the piezo devices can comprise a small diameter glass capillary surrounded by piezoelectric material which, if subjected to the application or removal of a voltage, results in constriction of the capillary tube. The constriction of the capillary tube causes ejection of a
30 droplet from the end of the capillary tube. Such piezo dispensing devices are highly accurate, but since they rely on surface wave excitation, they must be re-calibrated whenever the different fluids to be dispensed have significant differences in surface tension and/or viscosity. Pin-based dispensing technologies are less quantitative and suffer from high variability for different liquids. Fast solenoid technologies, as used

ATTORNEY DOCKET NO.21085.0035P1

5 herein, includes those which rely on a two step process in which, during the first step, a valve is open which allows a small amount of liquid to be pushed out of a small orifice at the end of the dispenser tip; and in the second step the valve is rapidly closed which freezes the motion of the liquid in the tube connecting the valve to the tip.

10 The dispensing which occurs with fast solenoid technology processes occurs when the kinetic energy of the rapidly moving liquid that is already outside the orifice exceeds the energy necessary to overcome the integrated surface energy of the liquid jet at the edge of the orifice. The use of fast solenoid technology provides significant advantages and is a preferred method of use within the current invention.

15 It is believed that fast solenoid dispensing will provide a means for accurately dispensing a wide range of liquids of different properties, as defined by their different surface tensions and viscosity values. For many solutions which can be used in the practice of the invention, this capability can be important as many prepared recipe solutions may have high viscosity and/or surface tension values.

20 Importantly, however, the use of the presently disclosed method of preparing recipe solutions in larger volumes and then redistributing these as needed greatly reduces the variability associated with differences in physical properties as the final recipe solutions prepared that will be redistributed in the smaller volumes, where differences in factors such as viscosity are so relevant, will be minimized.

25 Furthermore, the small differences that do occur can be minimized or adjusted for in the present invention. Specifically, devices of the present invention can be calibrated in accordance with the knowledge and understanding of those of skill in the art of automated or mechanical dispensing of fluids.

30 The calibration of individual dispenser tips used to dispense fluids can be used to provide uniform dispensing from an array of such tips. If the dispenser tips are to be calibrated, a machine vision system can be used. For example, the apparatus of the invention can be used to dispense sets of droplets of presumed volumes, the actual volumes dispensed can then be measured, and a calibration curve can be constructed for each individual dispensing tip system allowing for the

ATTORNEY DOCKET NO.21085.0035P1

5 proper adjustment of the dispensing apparatus. As is understood by those of skill in the art, these methods can encompass reiteration for optimization of accuracy and/or precision. Employing individual dispenser tip calibration during the operation of the apparatus for the practice of the invention provides for a low coefficient of variation amongst the tips and improves the overall accuracy and/or precision. These
10 advantages of higher precision and greater accuracy can be accomplished even when a variety and/or a multiplicity of dispenser tips are used. Methods of measuring volumes actually dispensed include visual inspection, determination of mass, and other means. For example, a solution of a light absorbing or fluorescent dye of known concentration can be dispensed, and the actual volume dispensed can then be
15 determined by use of absorbance or fluorimetric techniques.

In another aspect, the invention provides a method of conducting crystallization experiments in a droplet containing the protein and the recipe solutions, wherein the droplet is covered by oil of various compositions. The oil, when so used, can be to control a slow transport of water from the droplet through
20 the oil and into the surrounding environment. Slow transport provided by the coverage of the droplet by the oil allows for a slower loss of solvent and concomitantly a slower increase in the concentrations of the protein and other recipe ingredients than is possible when the droplet is not covered. This slower increase in protein and/or recipe ingredient concentration is preferable for orderly crystal
25 growth. Pure oils or mixtures of oils such as silicone and paraffin can be used to allow for rapid to very slow water diffusion rates from the droplet. In the present invention, layering oil over low volume droplets shortly after they are dispensed arrests the rapid evaporation of water, which, if left unchecked, would result in the loss of too much moisture from the droplets in too short a time. In addition, once
30 droplets are covered with oil, it is not necessary to cover each well with other means as rapidly as would otherwise be required. Thus oil coverage provides two distinct advantages, the first is unique to low volume dispensing (by arresting the rapid uncontrolled evaporation after dispensing), and the second is similar to previous experimental techniques in which oils are used to control the slow transport of water

ATTORNEY DOCKET NO.21085.0035P1

5 and the process kinetic rate. If a mixture of oils is used, the fraction of particular oils can be varied in accordance with necessary requirements to confer desired properties to the mixture of oils.

In an alternative embodiment, the use of oil to immediately cover droplets following their dispensing can be used in conjunction with crystallization trials
10 carried-out using the vapor diffusion process. In this case, oils that are highly permeable to water, such as silicone oil, are preferred as their use arrests the rapid evaporation of the small protein and recipe droplet, while allowing for the actual process kinetics to proceed in a rate similar to standard vapor diffusion experiments.

In certain embodiments, a mixture of oils can be used. For example, the oil can be a
15 mixture of silicone and paraffin oil. The oil can be chosen or the mixture of oils can be selected so as to modulate its characteristics to conform to a desired range of properties. In one preferred method, referred to as the modified vapor diffusion process, the protein solution is placed in a second well near a first well. The first well is filled with a recipe solution and the second well is filled with a mixture of the
20 protein solution and a smaller amount of the recipe solution than is present in the first well. A mixture of oils can be used to cover the first and/or second wells. The plate in which the first and second wells, respectively, or any number of first and second well pairs, are formed can be sealed with a clear cover or tape. Each well pair can be interconnected with a channel providing for vapor contact between each
25 pair of first and second wells.

In one embodiment of the present method, a plate used in the invention having first and second wells, can include a plate that has a series of more than one set of first and second wells. In a particular aspect, the plate as described can be fabricated from a standard 384 well microplate where a channel between first and
30 second wells in the same pair can be milled or otherwise formed by removal of a portion of the material between the adjacent wells such that the adjacent wells so connected by the channel are interconnected with one another when the plate is covered by a cover, by tape, or by other similar objects. Modulation of the degree of vapor communication between wells can be accomplished by variation in the size of

ATTORNEY DOCKET NO.21085.0035P1

5 the channel formed between each of the two interconnected wells. Variation in size of the channel can include variation in the depth, width or length of the channel. Alternatively, other devices or objects can be interposed in the functional channel between first and second wells of pairs. For example, a porous object, such as, but not limited to, a membrane or a sponge, can be interposed in the channel.

10 In another embodiment, recipe libraries can be prepared at much more dilute concentrations than those used by those skilled in the art. Similarly, protein solutions can be prepared at much more dilute concentrations. When either one, or both, of the solutions are prepared at more dilute concentrations, there are several benefits, including, but not limited to, more consistent and reliable dispensing of
15 solutions. The greater accuracy and precision of solution dispensing steps occurs both during recipe preparation from the bulk solutions (which are now more diluted), and during preparation of the experiments in the low volume range. The excess water, relative to what is necessary to promote crystallization, can be removed by evaporation. The rate at which the excess water is removed by
20 evaporation can be modified by adjusting the ratio of the water-permeable oils to the water-impermeable oils in the overlaying oil mixture, if the solution is overlayed with an oil or oil mixture. Alternatively, the droplet containing excess water can be covered initially with water-permeable oils that arrests the initial rapid evaporation following dispensing, but still provides for a reasonable loss of water over several
25 days thereby allowing the concentrations of protein and ingredients to reach levels significant to the crystallization process. Optionally, once evaporation has proceeded to a point where the concentrations of protein and/or ingredients are significant to crystallization, an additional quantity of oil can be added to the well containing the droplet. The additional quantity of oil can be of the same
30 composition and/or properties as the original oil used to overlay the droplet or it may differ. The use of the additional quantity of oil can be used to provide a slower evaporation rate such as is preferred for orderly crystal growth.

Alternatively, or in conjunction with an overlaying oil process, the rate of solvent evaporation can be altered by altering the humidity of the environment. In

ATTORNEY DOCKET NO.21085.0035P1

5 one aspect, the humidity of the environment can be increased to slow down the evaporation rate through the water permeable overlaying oil. In another aspect, the humidity of the environment can be decreased to increase the evaporation rate through the water permeable overlaying oil. Those skilled in the art can appreciate variations and combinations of the above embodiments to achieve the multiple
10 objectives of control over the initial evaporation rate, control of the release of excess water in the solution, and control over the slow process kinetics at the latter stage of the experiment.

In another embodiment of the batch crystallization method provided by the invention, the protein solution is dispensed into a well adapted for crystallization
15 prior to the recipe solution. The surface of the well can be adapted to modify its surface characteristics. While not being bound by theory, it is believed that the addition of protein solution to the well prior to addition of the recipe solution provides improved surface adhesion of the protein to the well's surface and a stable contact line between protein of the solution and the well surface. In contrast, when
20 the recipe solution is dispensed first, the droplet formed from both the recipe solution and the protein solution does not adhere to the well surface as well and the resultant combined droplet is typically pushed to the wall junction of the well upon addition of the overlaying oil.

In another embodiment, the ratio of the protein solution to the recipe solution
25 is varied across the crystallization array to provide an additional control variable during screening. The ratio of protein solution to recipe solution can be 1:99, 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, 50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 85:15, 90:10, 95:5, 99:1, wherein x:y designates x volume protein solution and y volume recipe solution.

30 In another aspect, the invention provides a device or a series of devices used to prepare solutions for crystallization screens, to prepare recipe libraries for crystallization screens, and to conduct the crystallization screens themselves. By way of illustration, such devices are shown in Figures 1-6. The system illustrated in Figures 1-6 comprises two distinct devices. The first device, shown in FIG. 1 and

ATTORNEY DOCKET NO.21085.0035P1

5 referred to as RecipeMaker 10, is used to prepare recipe solutions from bulk solutions. The RecipeMaker can also re-format recipe solutions from their initial containers (e.g., 2 milliliter volume wells in 96 deep-well plates) into smaller containers (e.g., 80 microliter in 384 deep-well plates). This particular example of a RecipeMaker is based on a commercially available robotic liquid handler (the
10 Microlab MPH-8, Hamilton Company, Reno, NV). In this example, the RecipeMaker 10 is outfitted with 8 dispensing tips that are movable via an XYZ directional arm 11 and 8 independently-controlled precision syringes, each having a volume of 500 milliliters. The arrangement of the component parts and functions of the RecipeMaker can be accomplished in many different ways, as known to those of
15 skill in the art, so long as the arrangement achieves the functionality described herein.

In one embodiment of the present invention, therefore, the input valve for each syringe can be connected to a computer-controlled rotating distribution valve that is also connected to 8 different supply tubes that lead to bottles containing
20 separate bulk solutions. This configuration, by providing access to 8 sets of 8 solutions, thus provides for access to 64 different bulk solutions. Those skilled in the art can appreciate that many other potential configurations can be constructed using different numbers of dispensing tips, syringes, distribution valves, and fluid delivery and transport means for accessing bulk solutions 12.

25 In another embodiment, the devices of the current invention are computer controlled and/or automated. A computer task file, containing the quantities desired from each solution used to form the recipes, can be read into the RecipeMaker control program. The program can then determine the bulk solutions to be connected via the distribution valves with the syringes, and thus to the dispensing
30 tips to prepare the recipe. In a preferred control program for the apparatus, the control program rearranges the sequence of dispensing solutions in such a way as to minimize the cleaning cycles required. As a significant amount of time and bulk solution costs are associated with the need to flush and/or clean the fluidics passages

ATTORNEY DOCKET NO.21085.0035P1

5 when switching from one bulk solution to another, this construction provides a significant advantage over any protocol which does not program the controller so as to minimize the number of wash steps. For example, when preparing recipes from 18 different bulk solutions, each typically containing up to 6 different bulk solutions, the control program used for this embodiment of the invention manages to restrict
10 the total number of system washes to 5 cycles and results in a significant savings in fluids and time.

Once the recipe solutions are prepared in the 1.5 milliliter plates, they can be sealed with commercially available heat seals (such as those available from Abgen, Inc., UK), bar-coded, and left to mix via liquid diffusion overnight. In alternative
15 embodiments, the recipes can be mixed using other techniques known to those of skill in the art, such as, but not limited to, vortexing, shaking, and mechanical mixing using magnetic beads and an external magnetic source. Once mixed, the recipe solutions can be stored as recipe libraries for different periods of time as is suitable for the solutions and the format in which they are stored. For example, it is
20 contemplated that the recipe libraries so formed can be stored in a manner analogous to combinatorial chemistry libraries that are used in the pharmaceutical industry for the discovery of new drugs. The RecipeMaker device, using a simple software command set available from the manufacturer, can also re-format a bulk recipe library from a first format, e.g., 96 well plates, into a second format, e.g., 384 well
25 plates. The latter format can be re-sealed, and can be used for the source of recipe solutions in subsequent embodiments of the invention. For example, the 384 well plate can be used as the recipe solution source in the next device. It is thus clear to those skilled in the art that other robotic liquid handlers with different hardware configurations, different numbers of wells in the recipe plates, and different recipe
30 storage arrangements can also be used.

The second device described herein for the purposes of illustrating certain aspects of the invention is shown in FIGS 2-6, and is referred to as NanoScreen 20. This device comprises a fast solenoid liquid dispensing system. The fast solenoid dispensing system can be movable using an XYZ directional transport stage 21. The

ATTORNEY DOCKET NO.21085.0035P1

5 device may have a deck 22 upon which the recipe plate 23 (which can contain a multiplicity of recipe solutions), a protein plate 24 (which can contain a multiplicity of protein solutions) and a crystallization plate 25, also referred to as a nano plate 25 (which can be used to contain a multiplicity of crystal screening condition reactions), can be situated. The deck 22 can also comprise other components which can be used
10 to perform ancillary operations including, but not limited to, quality control 26, calibration and washing stations 27.

NanoScreen's fast solenoid dispense system, shown in FIGS 2, 4, and 5, combines commercially available components (Innovadyne, Inc., Santa Rose, CA), that include a fast switching valve, 12 fast solenoid valves, and 12 dispenser tips 28.

15 Dispenser tips are mounted on a dispense head 29. The role of the fast switching valve is to rotate the fluidics passages for each tip between two positions. In the first position, each tip is fluidically connected to a computer-controlled syringe that is used to aspirate liquid solutions into the dispenser tip. The second position of the switching valve is use to connect each tip to a dedicated fast solenoid valve, that is
20 further connected to a constant pressure supply. Further aspects of this fluidics sub-system include rotating valves providing the use of two syringes to aspirate liquids from 12 different solutions into 12 different tips, and a computer-controlled pressure regulator valve to provide precise pressure control at the desired level. A separate system dispenses oil using 12 flexible dispensing tips that are connected to fast
25 solenoid valves (Lee, Inc. Westbrook, CT) and to a pressurized oil reservoir. The oil dispensing tips are mounted on an air cylinder providing a rapid motion in the Z-axis (vertically) between a top position used during motion of the entire dispense assembly, and a downward dispense position.

The deck area, shown in FIGS 2 and 3, contains 3 holders for standard plate
30 sizes: a recipe 384 well plate 23, a protein 384 well plate 24, and a crystallization 384 well plate 25. The recipe plate is a re-formatted 384 well plate that was prepared by RecipeMaker, the protein plate is a low-volume (40 microliter) 384 well plate, and the crystallization plate is a 384 well plate with a clear, flat bottom, and

ATTORNEY DOCKET NO.21085.0035P1

5 round wells. NanoScreen dedicates 2 of the 12 dispenser tips for dispensing the protein solutions. The protein plate thus contains protein solutions in only 2 of the wells, with other wells being used to return excess protein from the dispenser tips at the conclusion of the experiment run. It is also possible to use other plates, plate formats, or well configurations may be used. Many of these are commercially
10 available and will be recognized as being suitable for use in the current invention by those of skill in the art.

The primary considerations for choosing a recipe plate is its long-term chemical compatibility, while the principal reason for choosing a protein plate is the low well volume. The crystallization plate should preferably have a clear well
15 bottom to enable subsequent inspection for the appearance of crystals. Several additional plate formats specifically adapted for crystallization, and especially for vapor diffusion experiments with sitting drops, have recently become available (Corning, Inc., Acton, MA, and Greiner Bio-One, Germany). While the plates used and described herein for the sake of illustration are chosen for their compatibility
20 with the NanoScreen device, namely, the spacing (4.5 millimeter) for the NanoScreen dispenser tips, alternative plates can be used. Alternative dispenser tip spacings (e.g., 9 millimeter for 96 well plates) can be readily accommodated with the current design of the apparatus to provide for compatibility with other plates, such as the known types of commercial vapor diffusion plates (e.g., those supplied
25 by Corning, Inc.).

Other components and sub-systems positioned or carried on the deck include an area or station designated for quality control 26. In the context of a small volume dispenser, quality control is comprised of a periodic verification that no dispenser tip in the dispenser tip array is clogged. The quality control area includes a removable
30 glass surface, shown in FIG. 3, that is coated with a hydrophobic coating which results in a contact angle of approximately 90 degrees. A long range microscope equipped with a video camera is also provide, and is fixed to the dispenser head sub-system 29 and is thus movable with it using the same XYZ transport stage 21. The quality control station provides for the periodic dispensing of droplets from the

ATTORNEY DOCKET NO.21085.0035P1

5 dispenser tip array of desired liquid volumes on the glass surface, followed by automated machine vision inspection of these droplets.

Yet another area on the deck, shown in FIG 6, provides for the washing and drying of the dispenser tips. Since the NanoScreen device uses only two tips to dispense the protein solution, a wash station is provided for the protein tips 30. The wash station comprises a manifold into which the tips are lowered. A wash cycle is employed, in which the dispenser tips are externally saturated by strong jets of washing fluid, while simultaneously dispensing water through the tip and into the manifold. After a thorough cleaning of the tip's internal and external surfaces, a vacuum is provided to the manifold while the tips are kept in their inserted position to enable the rapid drying of the external surfaces. While the protein wash system has two holes in its manifold, the recipe wash system 31 has 10 such holes, with similar operations. The two wash systems provide for washing of the dispenser tips between aspirate/dispense cycles of the recipe solutions, and at the beginning and conclusion of the entire experiment for all of the dispenser tips 28.

20 The dispense, or dispenser, head system 29, together with the video camera (with the long range microscope) 33, is mounted on a commercially available XYZ directional transport stage (Parker-Hannifen, Cleveland, OH), as shown in FIGS. 2 and 5. The XYZ transport stage has a 12 inch range of motion in the X and Y (planar) axes, and a 3 inch range of motion in the Z (vertical) axis. Movement along each access is accurate to within 25 micrometers (positional accuracy).

The NanoScreen device can be computer controlled. For example, the control program can be written in Visual Basic, C++ (Microsoft Corporation, Redmond, WA) and LabView (National Instruments, Austin, TX), that runs under the Windows 98 operating system (Microsoft Corporation, Redmond, WA). The control program can provide capabilities for deck calibration (positional determination of all fixtures relative to the XYZ stage), dispenser tip calibration, a set of service routines that include, e.g., manual stage control, fluidics priming, manual dispensing, etc., and assay parameters setup, including, e.g., volume and

ATTORNEY DOCKET NO.21085.0035P1

5 sequence of dispensing, length of time for tip washing, automated quality control cycles, and other functions as are described herein or are needed.

With the NanoScreen device, dispenser tip calibration can be performed by repeatedly dispensing water at several volume settings on the quality control surface, followed by machine vision determination of the actual volume dispensed for each
10 setting using the video camera (with the long range microscope lens) 33. The control program then calculates a linear relationship between the volume setting and actual (measured) volumes, and stores the linear coefficients that are particular for each dispenser tip/valve fluidics system.

Each normal operation cycle is typically begun by priming the respective
15 dispenser tip fluidics system and validating the integrity of each tip/valve fluidic system. This is normally accomplished by dispensing a desired volume on the quality control area 26. This can then be followed by automated image acquisition and display of each dispensed droplet to the operator or to a software system. The recipe 23, protein 24, and crystallization plates 25 are then loaded on the deck either
20 manually or by a robotic operation. The cycle commences by the aspiration of protein solution from the protein wells into two dispenser tips. The tips are then moved to the quality control area and dispensing is performed, followed by machine vision inspection to determine the integrity of protein solution dispensing. Since protein solutions exhibit wide variations in their surface wetting, viscosity, and
25 surface tension characteristics, it is important to verify that each particular protein solution could be adequately dispensed. If dispensing proves to be difficult, and the system returns the protein dispenser tips to the protein plate and dispenses the protein solution back into neighboring wells for later retrieval, dilution, or other processing steps. As provided in the assay setup parameters, the system performs a
30 quality control cycle (dispensing followed by inspection) before commencing actual operations, periodically or as selected by the parameter settings.

Each cycle typically includes a recipe solution aspiration step, followed by protein solution dispense step, recipe solution dispense step, and the oil dispense

ATTORNEY DOCKET NO.21085.0035P1

5 step. The steps are performed in a block, defined as the number of dispenser tips in the recipe dispenser tip array (10 tips in the NanoScreen device depicted herein). The time required to dispense the protein and recipe solutions for each block is a few seconds, thus providing for the arrest of the fast evaporation thereof by immediate coverage of these solutions by the oil layer for each such block, the covering oil
10 being provided by the controlled dispensing of oil from the oil manifold 32. The recipe solution tip array is then moved to the recipe solution wash station 31, and the recipe solution tips washed internally and externally. The cycle is then repeated for the number of blocks specified in the assay setup parameters. The remaining protein solution is returned to the protein plate 24, and the entire dispenser tip
15 fluidics system is thereafter washed.

The NanoScreen and RecipeMaker devices/systems provide the means to perform the method of crystallization disclosed in the present invention. Improvements in the way the method of crystallization could be accomplished using modifications to or alternative devices/systems will be apparent to those skilled in
20 the art.

Experimental

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds,
25 compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless
30 indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

ATTORNEY DOCKET NO.21085.0035P1

5

Example 1

Recipe solutions were prepared by the system shown in Figure 1 using a statistical design of experiment methodology that combined ratios of ingredients from 18 types of bulk solutions. These solutions contained different buffers, salts, crystallization agents, and surfactants. The recipe solutions were prepared in 1.5 milliliter wells in 96 well plate and then sealed with tape. The ingredients were allowed to mix by diffusion over 24 hours, and then the recipe solutions were re-formatted into 384 plates. The recipe plates were placed on the deck of the dispenser shown in Figure 7, a concanavalin-A protein solution was placed on another plate on the deck, and an empty crystallization screen plate containing 384 wells was also placed on the deck. The dispenser, having a plurality of tips for aspirating and dispensing of the protein and recipe solutions, automatically prepared an array of crystallization experiments. Each well contained a unique recipe and the same protein solution, and was immediately covered with a layer of 80/20 %v/v paraffin to oil mixture. The dispensing volume of the protein solution was varied across the plate in three groups: 20, 40, and 60 nanoliter, and the recipe solutions volumes were also varied in three groups: 60, 40, and 20 nanoliter, such that the total droplet volume in each well was 80 nanoliter. The plates were then sealed with 3M Clear Tape and allowed to equilibrate at room temperature (about 21° C). The wells were periodically examined using a video microscope system. A total of 450 wells were prepared in three 384 plates. Several of the wells showed growth of small crystals after a few days of incubation. Figure 7 shows a typical well with crystals, where the droplet size was approximately 550 micrometers in diameter.

ATTORNEY DOCKET NO.21085.0035P1

5 Example 2

An experiment using the same conditions, solutions, and apparatuses was repeated with thaumatin instead of concanavalin-A. Several wells produced very large crystals, as shown in Figures 8 and 9, where the droplet diameters were typically 500 micrometers.

10

Example 3

A series of experiments using other proteins were conducted using the methods and devices of the present invention. The proteins listed in this example include both known and unknown proteins. The 2g1-1a and 9c9 proteins are
15 unknown proteins from the NIH Structural Genomics project. 4g49a and peIAE are unknown proteins provided by other researchers. The act_2 protein is a chymotrypsinogen. The W539A protein is a chitinase from an unknown organism. The pf glutamate dehydrogenase is a bacterial protein.

The results of these experiments, carried-out in accordance with the
20 teachings of the present invention, can be seen in Figure 10A-O. In Figure 10A, 2G1-1A was crystallized in 0.1 M Acetate (pH 4.5), 0.693 M NaCl, 12.37% PEGM5000, 0.01 M CaCl₂, and 0.05 M Arg-HCl at 4° C. In Figure 10B, 2G1-1A was crystallized in 0.1 M Tris (pH 8.5), 0.2 M NaOAc, and 30% PEG4000 at 22° C. In Figure 10C, 2g1-1a was crystallized in 0.1 M HEPES (pH 7.5), 0.289 M NaCl,
25 23.53% PEG4000, 6% glycerol, 0.01 M MgCl₂ and 0.05% BOG at 4° C. In Figure 10D, 4g49a was crystallized in 0.1 M HEPES (pH 7.5) and 1.5 M LiSO₄ at 22° C. In Figure 10E, 9c9 was crystallized in 0.1 M HEPES (pH 7.5), 0.264 sodium malonate, 4.7% PEG8000, 3% glycerol, 0.01 M MgCl₂ and 0.05 M ArgHCl at 4° C. In Figure 10F, 9c9 was crystallized in 0.1 M Bicine (pH 9), 0.11 M sodium citrate,
30 8.85% PEGM5000, 6% glycerol, 0.01 M CaCl₂, and 0.05 M ArgHCl at 14° C. In Figure 10G, act_2 was crystallized in 0.1 M Bicine (pH 8.3), 0.264 M ammonium sulfate, 4.79% PEG4000, 6% glycerol, 0.01 M MgCl₂ at 22° C. In Figure 10H, peIAE was crystallized in 0.1 M Bicine (pH 9), 0.479 M sodium citrate, 9.49% MPD, 0.01 M MgCl₂, and 0.05 M ArgHCl at 22° C. In Figure 10I, glutamate

ATTORNEY DOCKET NO.21085.0035P1

- 5 dehydrogenase was crystallized in 0.1 M HEPES (pH 7.5) and 4.3 M NaCl at 22° C. In Figure 10J, glutamate dehydrogenase was crystallized in 0.1 M MES (pH 6), 0.599 M sodium acetate, 10.65% PEG8000, and 0.05% BOG at 22° C. In Figure 10K, arsenate reductase was crystallized in 0.1 M Bicine (pH 8.3), 0.16 M ammonium sulfate, 14.3% MPD, 6% glycerol, 0.01 M MgCl₂, and 0.05 M ArgHCl at 14° C. In Figure 10L, arsenate reductase was crystallized in 0.1 M MES (pH 6), 0.25 M sodium malonate, and 20.06% PEGM5000 at 22° C. In Figure 10M, W539A was crystallized in 0.1 M HEPES (pH 7.5), 0.289 M KSCN, 23.1% PEG8000 and 0.05% BOG at 4° C. In Figure 10N, W539A was crystallized in 0.1 M HEPES (pH 7.5), 0.25 M NaCl, 19.97% PEG8000, 3% glycerol and 0.05 M ArgHCl at 14° C. In Figure 10O, 9c9 was crystallized in 0.1 MES (pH 6), 0.2 M sodium malonate, 16.26% PEG4000, 3% glycerol, and 0.01% CaCl₂ at 14° C.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

ATTORNEY DOCKET NO.21085.0035P1

What is claimed is:

- 1) A method for screening protein crystal growth conditions comprising the steps of:
 - (a) providing a microarray with a plurality of wells in the microarray;
 - (b) dispensing a volume of protein solution containing a protein into at least one of the wells;
 - (c) dispensing a recipe solution into at least one of the wells, wherein the recipe solution was prepared from bulk ingredients prior to being dispensed into the wells and was stored in a plurality of containers or in a second multiwell plate;
 - (d) subjecting the protein solution, or a combination solution formed by the combination of the protein solution with the recipe solution, to environmental conditions effective to form protein crystals; and
 - (e) observing the growth of protein crystals or precipitation of protein.
- 2) The method of claim 1, wherein a solution selected from the group consisting of the protein solution, the recipe solution and the combination solution is covered by a first oil.
- 3) The method of claim 1, wherein the recipe solutions are prepared in a volume of greater than or equal to 1 microliter.
- 4) The method of claim 1, wherein the recipe solutions are prepared in a volume of less than 5 milliliters.
- 5) The method of claim 1, wherein the recipe solutions are mixed by a method selected from the group consisting of vortexing, use of magnetic stirring elements, agitation and shaking.

ATTORNEY DOCKET NO.21085.0035P1

- 6) The method of claim 1, wherein fractions of the recipe solutions are transferred from the containers or the second multiwell plate into further containers or further multiwell plates.
- 7) The method of claim 1, wherein the dispensing of the protein or the recipe solutions is conducted using a dispensing device comprising fast solenoid technology.
- 8) The method of claim 7, wherein the dispensing device comprises the use of adjustable constant back pressure.
- 9) The method of claim 7, wherein the dispensing device uses fast solenoid technology with syringe dispensing.
- 10) The method of claims 7, wherein the dispensing device is adapted to use a dispenser tip.
- 11) The method of claim 1, wherein the recipe solutions are prepared from the bulk ingredient using a dispensing device comprising fast solenoid technology.
- 12) The method of either claim 11, wherein the dispensing device comprises the use of adjustable constant back pressure.
- 13) The method of claim 11, wherein the dispensing device uses fast solenoid technology with syringe dispensing.
- 14) The method of claims 11, wherein the dispensing device is adapted to use a dispenser tip.

ATTORNEY DOCKET NO.21085.0035P1

- 15) The method of claim 1, wherein the dispensing of the protein or the recipe solutions is conducted using a dispensing device comprising piezo technology.
- 16) The method of claims 15, wherein the dispensing device is adapted to use a dispenser tip.
- 17) The method of claim 1, wherein the recipe solutions are prepared from the bulk ingredient using a dispensing device comprising piezo technology.
- 18) The method of claims 17, wherein the dispensing device is adapted to use a dispenser tip.
- 19) The method of claim 1, wherein the dispensing device is adapted to use a dispenser tip and wherein the tip is calibrated for fluids which the dispensing device is used to dispense the fluids dispensed.
- 20) The method of claim 19, wherein the tip is calibrated by; i) dispensing a designated quantity of the fluid; ii) determining the actual delivered volume; and iii) adjusting indicated values for designated quantity of fluid to correspond with the actual volume delivered.
- 21) The method of claim 20, wherein the volume is determined by visually determining the actual delivered volume.
- 22) The method of claim 20, wherein the volume is determined by quantifying a detectable agent contained in the designated quantity of the fluid, wherein the concentration of the detectable agent is known.
- 23) The method of claim 22, wherein the detectable agent is a dye that can be detected by quantifying its absorbance or by its fluorescence.

ATTORNEY DOCKET NO.21085.0035P1

- 24) A method for screening protein crystal growth conditions comprising the steps of:
- (a) providing an array of at least two wells;
 - (b) dispensing a volume of protein solution containing a protein into at least one of the wells;
 - (c) dispensing a recipe solution into at least one of the wells, wherein the recipe solution was prepared from bulk ingredients prior to being dispensed into the wells and was stored in at least one of a plurality of containers or in a second multiwell plate;
 - (d) subjecting the protein solution, or a combination solution formed by the combination of the protein solution with the recipe solution, to environmental conditions effective to form protein crystals; and
 - (e) observing the growth of protein crystals or precipitation of protein.
- 25) The method of claim 24, wherein a solution selected from the group consisting of the protein solution, the recipe solution and the combination solution is covered by a first oil.
- 26) The method of claim 25, wherein the first oil is water-permeable.
- 27) The method of claim 26, further comprising an additional layer of oil, wherein the additional layer of oil overlays the first oil.
- 28) The method of claim 27 wherein the additional layer of oil is added after the first oil is added.
- 29) The method of claim 28 wherein the additional layer of oil is added after the volume of the solution over which the additional layer of oil is added has been reduced by evaporation.

ATTORNEY DOCKET NO.21085.0035P1

- 30) The method of claim 29 wherein the additional layer of oil is less permeable to water than the first oil.
- 31) The method of claim 25, wherein the first oil is water-impermeable.
- 32) The method of claim 31, further comprising an additional layer of oil, wherein the additional layer of oil overlays the first oil.
- 33) The method of claim 32, wherein the additional layer of oil is added after the first oil is added.
- 34) The method of claim 33, wherein the additional layer of oil is added after the volume of the solution over which the additional layer of oil is added has been reduced by evaporation.
- 35) The method of claim 34, wherein the additional layer of oil is less permeable to water than the first oil.
- 36) The method of claim 25, wherein step (d) comprises subjecting the solutions to a defined or selected humidity or range of humidities.
- 37) The method of claim 24, wherein a combination solution is formed in the wells of a multiwell plate.
- 38) The method of claim 37, wherein the protein solution is dispensed into the wells of the multiwell plate prior to the recipe solution.
- 39) The method of claim 24, wherein the conditions effective to form crystals must evaporate greater than 10% of the protein or the combination solution.

ATTORNEY DOCKET NO.21085.0035P1

- 40) The method of claim 39, wherein the conditions effective to form crystals must evaporate greater than 20% of the protein or the combination solution.
- 41) The method of claim 40, wherein the conditions effective to form crystals must evaporate greater than 30% of the protein or the combination solution.
- 42) The method of claim 41, wherein the conditions effective to form crystals must evaporate greater than 40% of the protein or the combination solution.
- 43) The method of claim 42, wherein the conditions effective to form crystals must evaporate greater than 50% of the protein or the combination solution.
- 44) The method of claim 43, wherein the conditions effective to form crystals must evaporate greater than 60% of the protein or the combination solution.
- 45) The method of claim 44, wherein the conditions effective to form crystals must evaporate greater than 75% of the protein of the combination solution.
- 46) The method of claim 24, wherein at least a pair of the protein and the recipe solutions are in vapor-vapor contact with each other.
- 47) The method of claim 46, wherein the protein solution, the recipe solution or both the protein solution and the recipe solution are overlaid with an oil.
- 48) A crystallization system, comprising a first recipe solution preparation and storage sub-system used to prepare a plurality of recipe solutions for storage until said recipe solutions are required for use within the crystallization system, and a second aspiration and dispensing sub-system used to aspirate and dispense a

ATTORNEY DOCKET NO.21085.0035P1

plurality of protein solutions and of said recipe solutions, respectively, into a plurality of wells.

- 49) The recipe preparation and storage sub-system of claim 48, comprising at least one fluid dispenser tip, at least one syringe, and at least one distribution valve, wherein the total number of fluid dispenser tips provided is less than the total number of a plurality of available bulk solutions from which the recipe solutions are prepared, and wherein the total number of distribution valves used to connect different ones of said bulk solutions to the at least one fluid dispenser tip and the at least one syringe, respectively, is minimized.
- 50) The recipe preparation and storage sub-system of claim 48, further comprising a plurality of multi well plates, each said well plate having a volume ranging from about 10 microliter to about 10 milliliters.
- 51) A crystallization experiment preparation system, comprising:
- a) a dispense head provided with plurality of fluid dispenser tips;
 - b) a video camera and a long range microscope each carried on said dispense head;
 - c) a stage constructed and arranged to move the dispense head, the video camera, and the long range microscope together in the X, the Y, and the Z directions, respectively;
 - d) a deck area positioned with respect to the stage, said deck area having at least one fixture for receiving at least one recipe plate, at least one protein plate, at least one crystallization plate, a quality control area, and a wash system, respectively, thereon.
- 52) A crystallization experiment preparation system, comprising:
- a) a dispense head provided with plurality of fluid dispenser tips;

ATTORNEY DOCKET NO.21085.0035P1

- b) a video camera and a long range microscope each carried on said dispense head;
 - c) a deck area positioned with respect to the dispense head, said deck area having at least one fixture for receiving at least one recipe plate, at least one protein plate, at least one crystallization plate, a quality control area, and a wash system, respectively, thereon;
 - d) wherein the dispense head, the video camera, and the long range microscope are held together in a stationary position with respect to a deck area; and
 - e) wherein the deck area is constructed and arranged to be moved with respect to the dispense head, the video camera, and the long range microscope by a stage movable in the X, the Y, and the Z, directions, respectively.
- 53) A crystallization experiment preparation system, comprising:
- a) a dispense head provided with plurality of fluid dispenser tips;
 - b) a video camera and a long range microscope each carried on said dispense head;
 - c) a first stage on which the dispense head is positioned, said first stage being constructed and arranged to move the dispense head in the Z direction;
 - d) a deck area positioned with respect to the dispense head, said deck area having at least one fixture for receiving at least one recipe plate, at least one protein plate, at least one crystallization plate, a quality control area, and a wash system, respectively, thereon; and
 - e) a second stage adapted to carry said deck area in the X and the Y directions, respectively.
- 54) The crystallization experiment preparation system of claim 53, said dispense head further comprising an aspiration and dispensing system.

ATTORNEY DOCKET NO.21085.0035P1

- 55) The crystallization experiment preparation system of claim 54, said aspiration and dispensing system comprising a constant pressure fast solenoid.
- 56) The crystallization experiment preparation system of claim 54, said aspiration and dispensing system comprising a piezo device.
- 57) The crystallization experiment preparation system of claim 54, said aspiration and dispensing system comprising a syringe-driven fast solenoid.
- 58) The crystallization experiment preparation system of claim 53, further comprising a dispenser tip fluidics system, said dispenser tip fluidics system comprising a plurality of fluid dispenser tips.
- 59) The crystallization experiment preparation system of claim 58, wherein each dispenser tip of the tip fluidics system is individually calibrated.
- 60) The crystallization experiment preparation system of claim 59, wherein each said individually calibrated dispenser tips has an individual calibration curve used to dispense a measured and predetermined volume of a protein solution or a recipe solution, respectively, therethrough.
- 61) The crystallization experiment preparation system of claim 53, further comprising at least one droplet array, each said at least one droplet array comprising at least one protein solution and at least one recipe solution, respectively, prepared in blocks of protein and recipe solutions, respectively.
- 62) The crystallization experiment preparation system of claim 61, wherein the total time required to prepare each said protein solution and recipe solution block, respectively, ranges from about one to about sixty seconds.

1/10

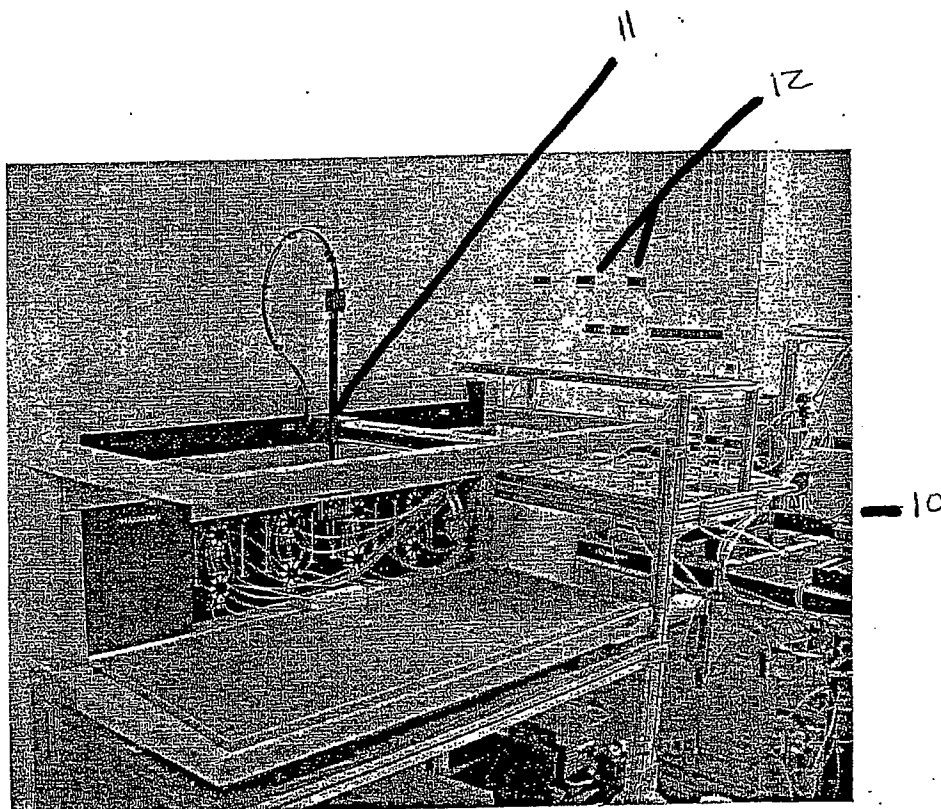


FIG. 1

2/10

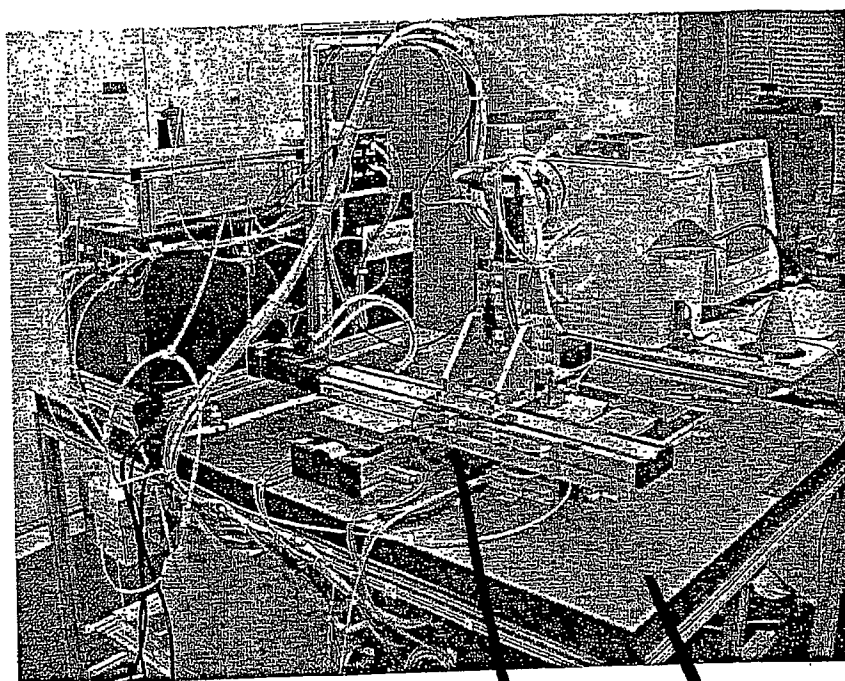


FIG. 2

3/10

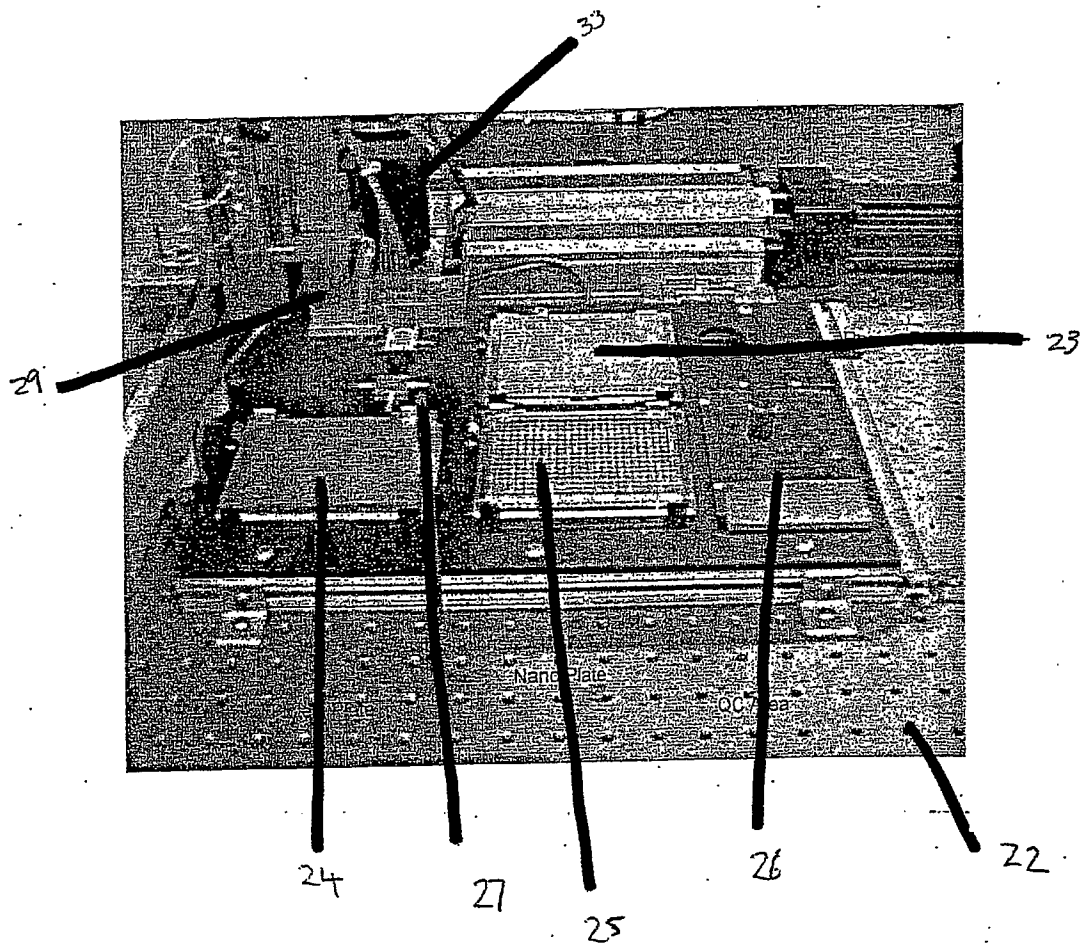


FIG. 3

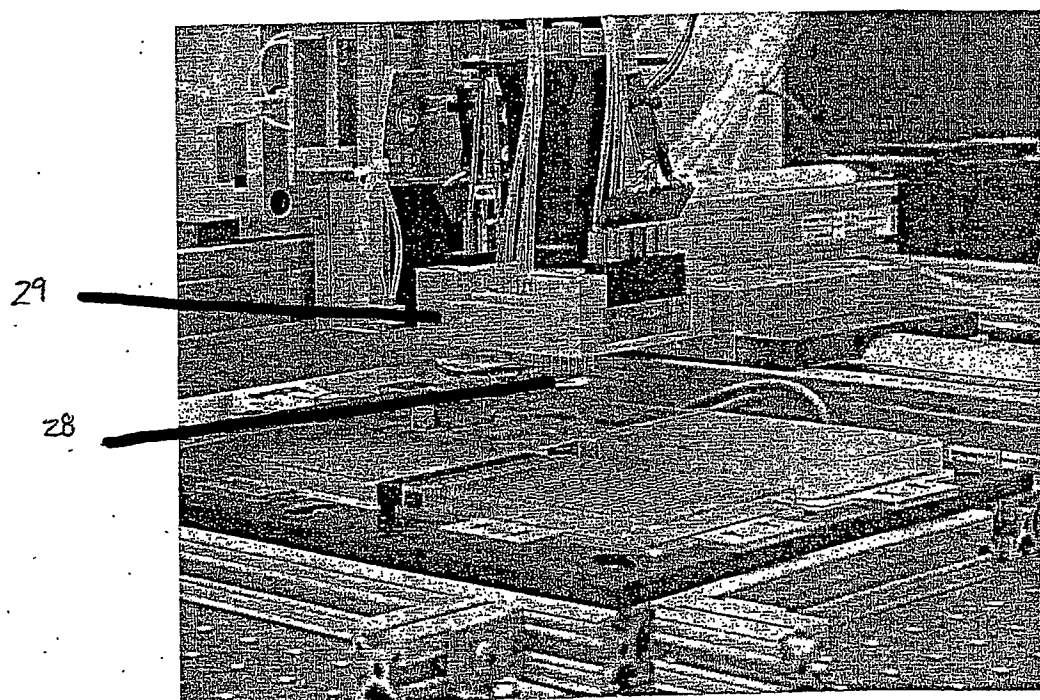


FIG. 4

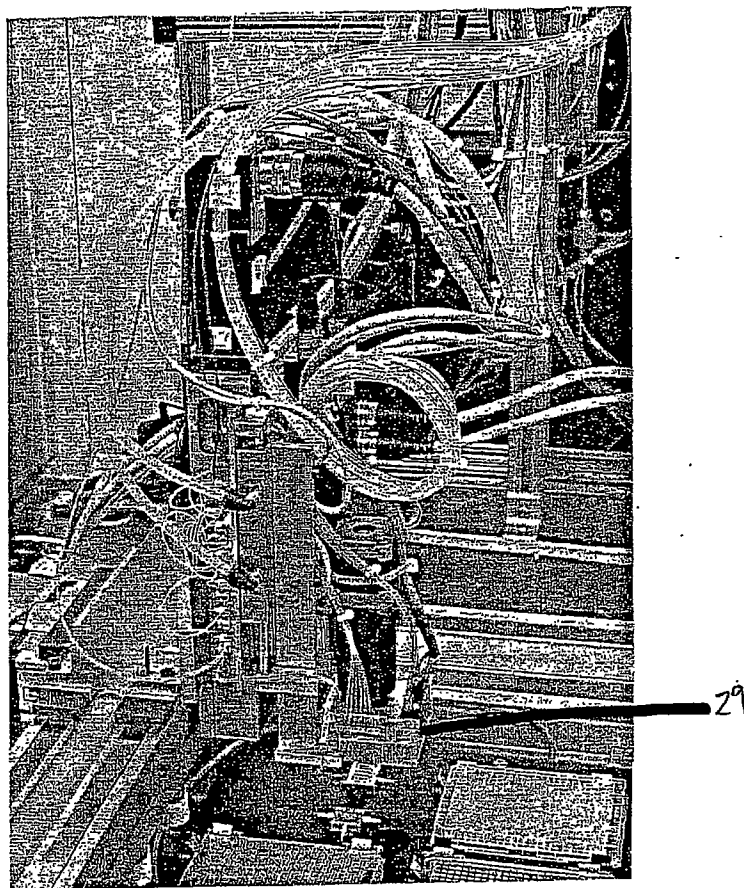


FIG. 5

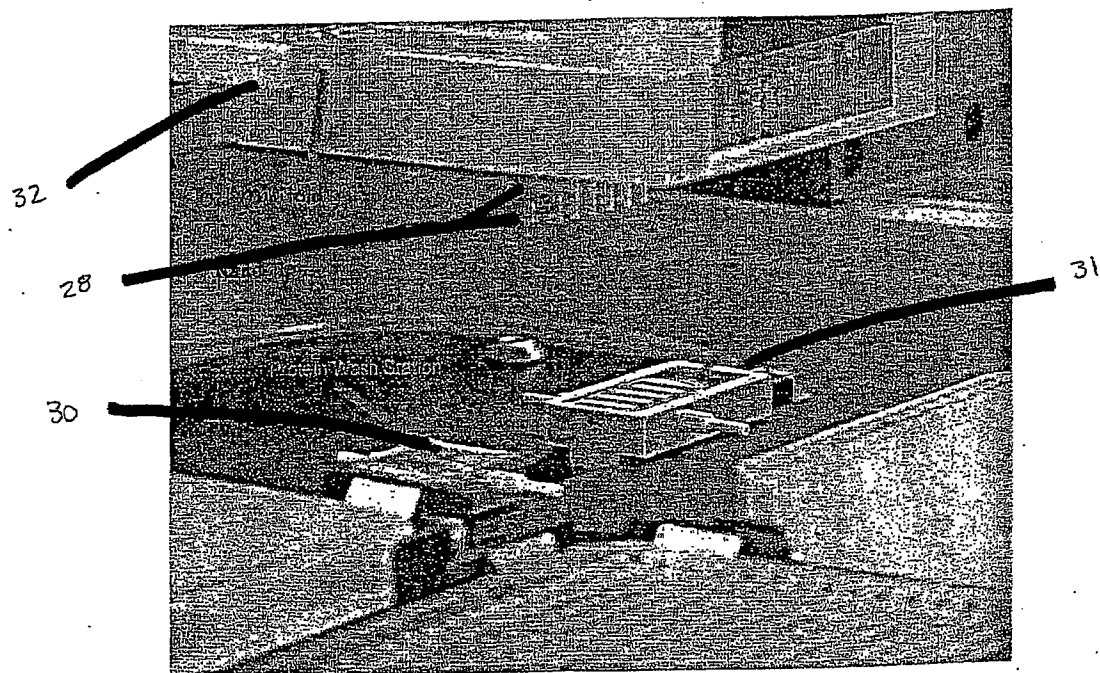


FIG. 6

7/10

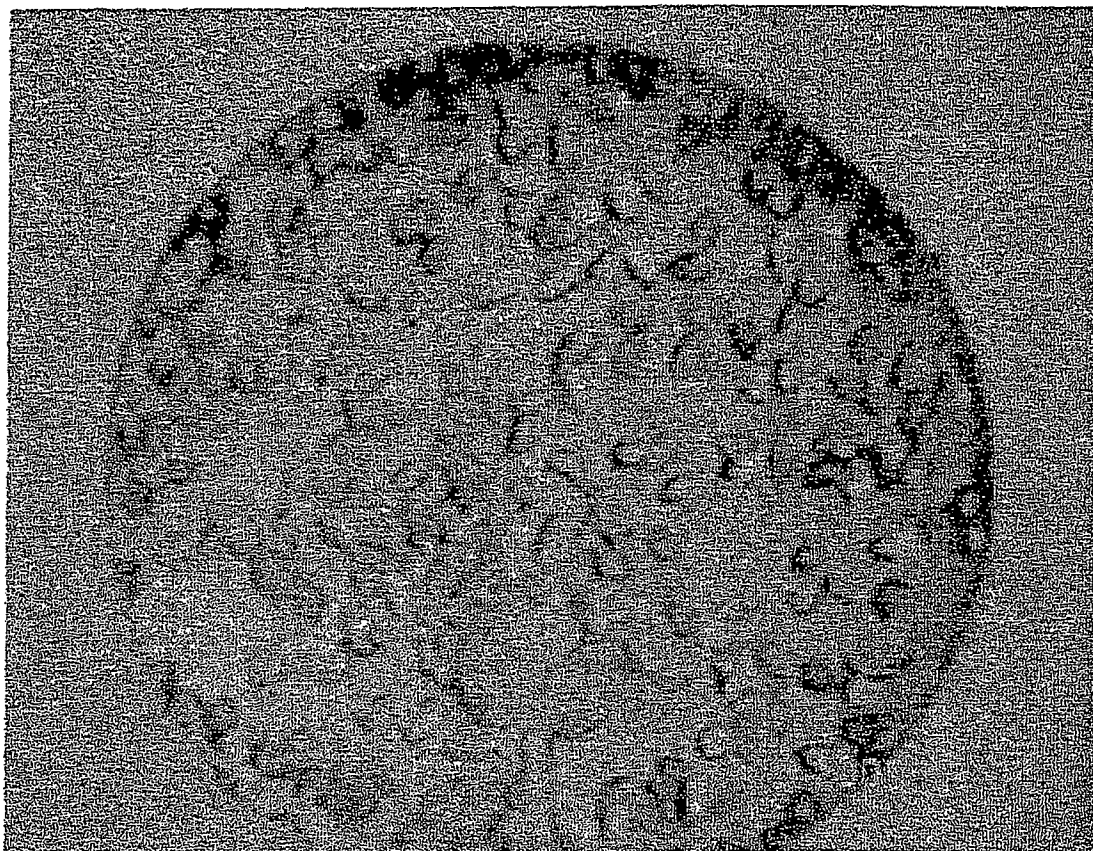


FIG. 7

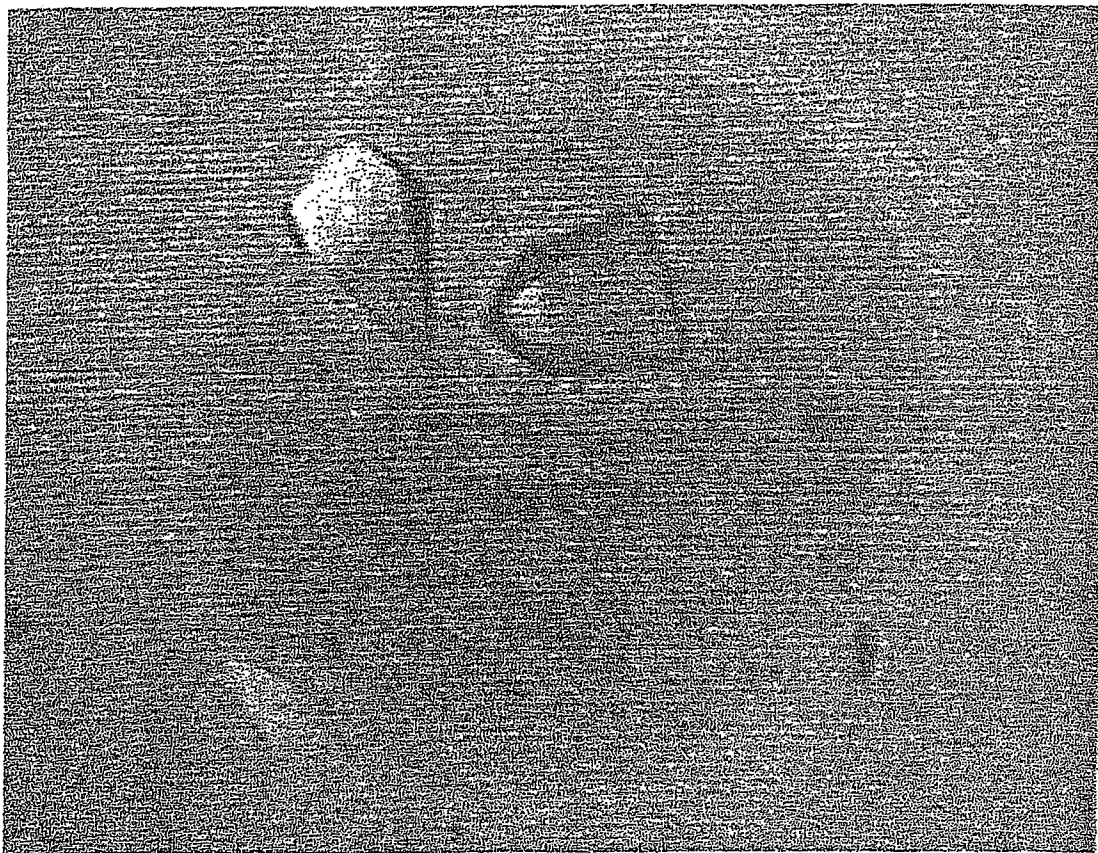


FIG. 8



FIG. 9

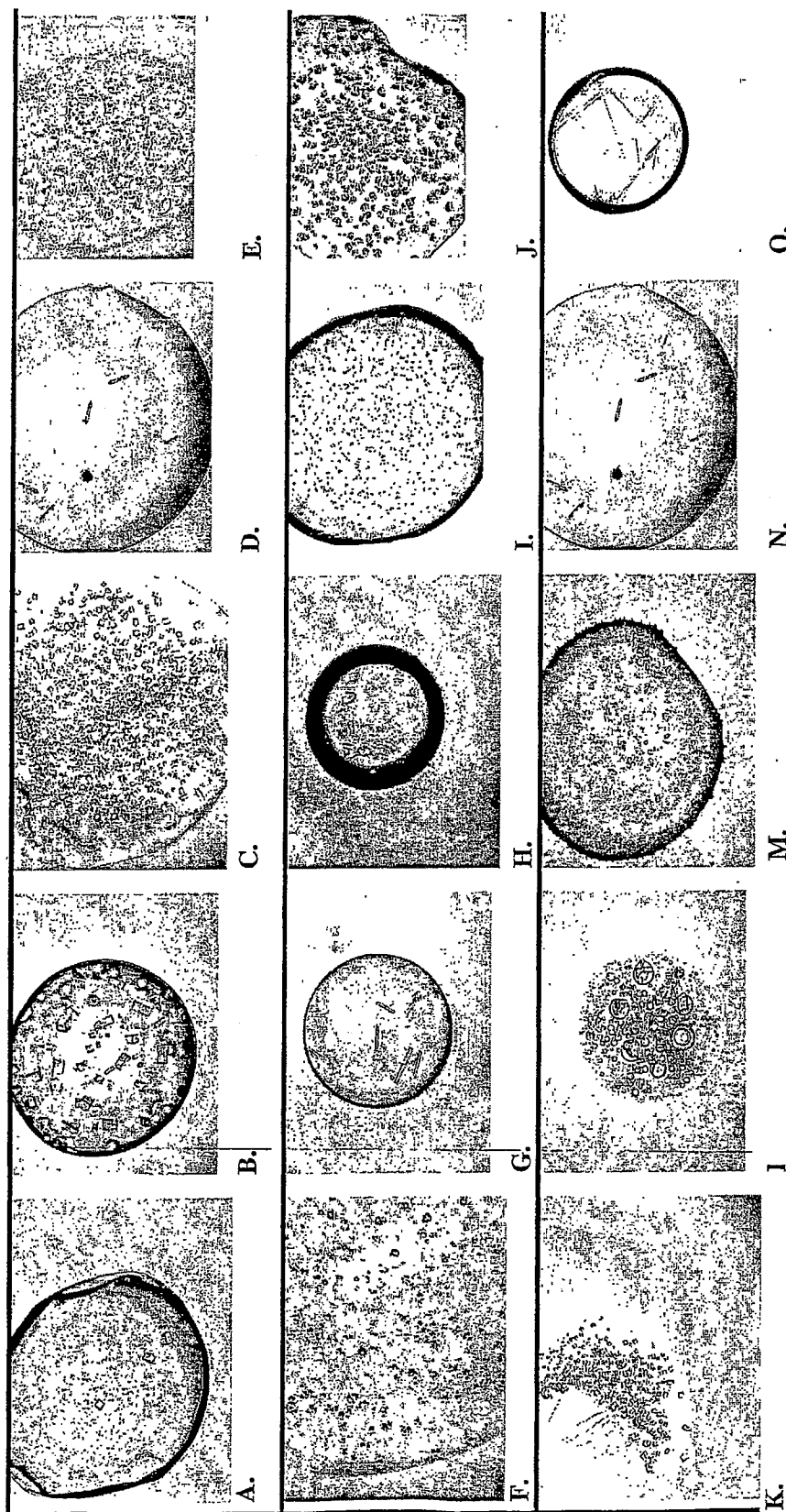


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/33890

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : B01D 9/00; G01N 1/00 33/00, 21/76

US CL : 23/295, 296; 436/86, 172, 174, 800

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 23/295, 296; 436/86, 172, 174, 800

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/60345 A1 (UNIVERSITY OF ALABAMA AT BIRMINGHAM RESEARCH FOUNDATION) 12 October 2000, claims 1-38 and pages 2-19.	1, 3-7, 24-25, 36-46
Y	US 6,291,192 B1 (PANTOLIANO et al.) 18 September 2001, columns 5-7 and 33-63 and claims 7-10.	1-14, 17-23 and 48-62
Y	US 6,268,158 B1 (PANTOLIANO et al.) 31 July 2001, Claims 1-18, Examples 1, 7-10, 12 and 18, and columns 43 and 60.	1-47
Y	US 6,296,673 B1 (SANTARSIERO et al.) 02 October 2001, columns 2-6 and 17-18.	1, 7-18 and 48-62
Y	US 5,221,410 A (KUSHNER et al.) 22 June 1993, Figures 1-7, columns 5-10, and claims 1-6.	1-20 and 24-47

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

27 December 2002 (27.12.2002)

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

Box PCT

Washington, D.C. 20231

Facsimile No. (703)305-3230

Date of mailing of the international search report

13 JAN 2003

Authorized officer

Samuel Wei Liu

Telephone No. 703-308-0196